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2) Young, J. W., Szabolcs, P. & Moore, M. A. S. Identification of dendritic cell colony-forming units among normal CD4+

bone marrow progenitors that are expanded by c-kit-ligand and yield pure dendritic cell colonies in the presence of granulocyte/macrophage colony-stimulating factor and tumor necrosis factor. J. Exp. Med. 182, 1111-1120 (1995). |

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Art Unit 1647  
CM1 10B-11  
703-308-1793

# Identification of Dendritic Cell Colony-forming Units among Normal Human CD34<sup>+</sup> Bone Marrow Progenitors That Are Expanded by *c-kit*-ligand and Yield Pure Dendritic Cell Colonies in the Presence of Granulocyte/Macrophage Colony-stimulating Factor and Tumor Necrosis Factor $\alpha$

By James W. Young,\*‡§ Paul Szabolcs,\*<sup>1</sup> and Malcolm A. S. Moore¶

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## Summary

Several cytokines, especially granulocyte/macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), have been identified that foster the development of dendritic cells from blood and bone marrow precursors in suspension cultures. These precursors are reported to be infrequent or to yield small numbers of dendritic cells in colony-forming assays. Here we readily identify dendritic cell colony-forming units (CFU-DC) that give rise to pure dendritic cell colonies. Human CD34<sup>+</sup> bone marrow progenitors were expanded in semi-solid cultures with serum-replete medium containing *c-kit*-ligand, GM-CSF, and TNF- $\alpha$ . The addition of TNF- $\alpha$  to GM-CSF did not alter the number of typical GM colonies but did generate pure dendritic cell colonies that accounted for ~40% of the total colony growth. When the two distinct types of colonies were plucked from methylcellulose and tested for T cell-stimulatory activity in the mixed leukocyte reaction, the potency of colony-derived dendritic cells exceeded that of CFU-GM progeny from the same cultures by at least 1.5–2 logs. Immunophenotyping and cytochemical staining of the CFU-DC-derived progeny was also characteristic of dendritic cells. Other myeloid cells were not identified in these colonies. The addition of *c-kit*-ligand to GM-CSF- and TNF- $\alpha$ -supplemented suspensions of CD34<sup>+</sup> bone marrow cells expanded CFU-DCs almost 100-fold by 14 d. We conclude that normal human CD34<sup>+</sup> bone marrow cells include substantial numbers of clonogenic progenitors, distinct from CFU-GMs, that can give rise to pure dendritic cell colonies. These CFU-DCs can be expanded for several weeks by *in vitro* culture with *c-kit*-ligand, and their differentiation requires exogenous TNF- $\alpha$  in addition to GM-CSF. We speculate that this dendritic cell-committed pathway may in the steady state contribute cells to the epidermis and afferent lymph, where dendritic cells are the principal myeloid cell type, and may increase the numbers of these specialized antigen-presenting cells during T cell-mediated immune responses.

Cytokines like GM-CSF and TNF- $\alpha$  are known to support the development of dendritic cells from progenitors in blood and marrow (1–8). We have found that the addition of *c-kit*-ligand (KL)<sup>1</sup> substantially increases the

yield of dendritic cells in suspension cultures relative to the starting number of human CD34<sup>+</sup> bone marrow cells. Nevertheless, these myeloid progeny are heterogeneous, dendritic cells constitute only ~10–15% of the total, and monocytes constitute the remaining class II MHC positive cells (8). The monocyte progeny are plastic adherent, are CD14<sup>+</sup>, and are weak stimulators of the MLR, whereas the mature dendritic cells are nonadherent, are CD14<sup>+</sup>, and are potent MLR stimulators. In prior reports of colony-forming assays, dendritic cells have represented a minor fraction of the clonogenic progeny as well. Inaba et al. identified a

Portions of this work were presented in abstract form at the Keystone Symposium, Dendritic Cells: Antigen-presenting Cells of T and B Lymphocytes, in Taos, NM, 10–16 March 1995.

<sup>1</sup>Abbreviations used in this paper: BMNC, bone marrow mononuclear cells; CFU-DC, dendritic cell CFU; CFU-GM, granulocyte/macrophage CFU; epo, erythropoietin; KL, *c-kit*-ligand.

class II MHC negative precursor in murine bone marrow that yielded mixed myeloid colonies after exposure to GM-CSF (5), including 0.5–1.5% highly stimulatory dendritic cells. Reid and colleagues first reported the existence of a GM-CSF/IL-3 and TNF- $\alpha$  responsive progenitor among human CD34<sup>+</sup> cells that could yield colonial progeny with some of the features of dendritic cells (2). These CD34<sup>+</sup>-derived colonies were mixed, however, and included additional cells with macrophage characteristics. Dendritic cells are not as easily distinguished from monocytes/macrophages in the human as they are in the mouse, and it has been difficult to generate human dendritic cell colonies of sufficient frequency and purity to confirm the functional as well as phenotypic and/or morphologic identity of these progeny as true dendritic cells.

We therefore undertook colony-forming assays using human CD34<sup>+</sup> bone marrow cells and various combinations of KL, GM-CSF, and TNF- $\alpha$  in serum-replete, semi-solid media. Morphologically distinct colony types were identified, plucked, and compared cytochemically, phenotypically, and functionally. Recruitment and expansion of CFUs and the contribution of KL were determined in secondary clonogenic assays after serial expansions in bulk suspension culture. This approach has identified a novel hematopoietic pathway for the clonogenic differentiation of pure dendritic cells from dendritic cell CFUs (CFU-DC) that are roughly comparable in frequency with, but distinct from, granulocyte/macrophage CFUs (CFU-GM) among human CD34<sup>+</sup> bone marrow progenitors.

## Materials and Methods

**Culture Media and Cytokines.** CD34<sup>+</sup> bone marrow progenitors were cultured in IMDM (GIBCO BRL, Gaithersburg, MD) with 50  $\mu$ g/ml gentamicin (Sigma Chemical Co., St. Louis, MO),  $7.3 \times 10^{-5}$  M monothio glycerol (Sigma Chemical Co.), 20% FCS (heat inactivation not required; Gemini Bioproducts, Calabasas, CA), and exogenous cytokines as indicated. MLR assays for evaluating mature, differentiated progeny used RPMI 1640 (GIBCO BRL) with 10 mM Hepes (Sigma Chemical Co.), 1 mM glutamine (GIBCO BRL),  $5 \times 10^{-5}$  M 2-ME (Eastman Kodak Co., Rochester, NY), penicillin (100 U/ml)-streptomycin (100  $\mu$ g/ml) (GIBCO BRL), and 10% heat-inactivated normal human serum. The recombinant human cytokines used were as follows: 20 ng/ml KL or stem cell factor (Amgen Biologicals, Thousand Oaks, CA); 100 ng/ml GM-CSF (Sandoz Pharmaceuticals Corp., East Hanover, NJ); 10 ng/ml TNF- $\alpha$  (Genentech, Inc., San Francisco, CA); 50 ng/ml IL-3 (Amgen Biologicals); 20 ng/ml IL-6 mutein (mutant of recombinant native protein; Imclone Systems, Inc., New York); and 5 U/ml erythropoietin (epo; Amgen Biologicals).

**Isolation of Human CD34<sup>+</sup> Bone Marrow Progenitors.** Small aliquots of bone marrow were obtained from normal donors already undergoing harvesting for allogeneic transplantation. CD34<sup>+</sup> cells were isolated by positive selection of anti-CD34 (clone 11.1.6, IgG1; Oncogene Science Inc., Uniondale, NY)-opsonized bone marrow mononuclear cells (BMMNC), using sheep anti-mouse IgG1 (Fc-specific) immunomagnetic beads (Dynal, Oslo, Norway) (8, 9). After 16–20 h of incubation at 37°C in IMDM–20%

FCS, the beads detached and CD34<sup>+</sup> cells were harvested. The yield was ~1% of the starting BMMNC, and further selection of more primitive progenitors was not undertaken. CD34<sup>+</sup> progenitors isolated from either unmodified or partially T cell-depleted, soybean agglutinin-negative (10, 11) bone marrow gave similar results.

**Culture of Human CD34<sup>+</sup> Progenitors in Suspension and Colony-forming Assays.** Colony-forming assays were performed in triplicate starting with  $1-2 \times 10^3$  CD34<sup>+</sup> cells in 1-ml cultures. Semi-solid medium consisted of either 0.36% agarose (FMC Corp. Bioproducts, Rockland, ME) and complete IMDM–20% FCS, or 1.2% methylcellulose (FMC Corp. Bioproducts) and complete IMDM–30% FCS with additional supplements of  $5 \times 10^{-5}$  M 2-ME,  $2 \times 10^{-3}$  M glutamine, and  $2.5-5 \times 10^{-6}$  M hemin. Exogenous cytokine supplements have been indicated for each experiment. CFUs were quantified by counting colonies of  $\geq 50$  cells after 12–14 d of culture. Baseline CFUs were enumerated in primary colony cultures established at the time of CD34<sup>+</sup> progenitor isolation. Recruitment and expansion of CFUs were assessed in secondary clonogenic assays after serial expansions in suspension culture. Suspension cultures of CD34<sup>+</sup> bone marrow cells were initiated with complete IMDM–20% FCS at an initial density of  $2 \times 10^5/5$  ml in 35-mm tissue culture wells. All cultures were maintained at 37°C in humidified 5% CO<sub>2</sub>.

**Cytochemical and Immunophenotypic Staining of Cytospin Preparations.** Individual colonies of a single morphologic type were plucked from methylcellulose after 12–14 d of culture under direct inspection by inverse-phase microscopy in a laminar air flow hood. The cells were resuspended in IMDM–20% FCS without cytokines and washed. Cells were resuspended in RPMI 1640–10% normal human serum and warmed in a 37°C water bath, after which  $0.5-1 \times 10^4$  cells/100  $\mu$ l were cytocentrifuged onto glass slides (900 rpm for 3 min; Cytospin 2; Shandon Inc., Pittsburgh, PA), air dried, and acetone fixed. Morphology was assessed by Wright-Giemsa staining. Cytochemical evaluation included staining for nonspecific esterase ( $\alpha$ -naphthyl butyrate esterase) (12), specific esterase (chloroacetate esterase; Sigma Chemical Co.), myeloperoxidase (Sigma Chemical Co.), and acid phosphatase (Sigma Chemical Co.). Colonies were also stained cytochemically in situ after methanol dehydration and acetone fixation of agarose plates. Immunophenotyping used mAbs to HLA-DR/IDQ (9.3C9 subclone of 9.3F10, IgG<sub>2</sub>; HB 180), CD1a (OKT6; IgG<sub>1</sub>; CRL 8020), and CD14 (3C10, IgG<sub>2</sub>; TIB 228) (all from American Type Culture Collection, Rockville, MD). These were followed by biotinylated goat anti-mouse Ig (No. 4753, Tago, Inc., Burlingame, CA) and a preformed avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories, Inc., Burlingame, CA). Positive staining was detected using diaminobenzidine tetrahydrochloride (Polysciences, Inc., Warrington, PA) as the chromogenic enzyme substrate.

**PBMC and Enrichment of Blood Dendritic Cells, Monocytes, and T Lymphocytes (13–16).** Mononuclear leukocyte subpopulations were prepared according to previously published procedures, using Ficoll-Hypaque-separated PBMC from normal leukocyte concentrates (Greater NY Blood Center, New York). Primary T cells were rosetted with neuraminidase-treated sheep erythrocytes followed by elution over nylon wool columns. Mature blood dendritic cells were purified from the nonadherent, T cell-depleted, sheep erythrocyte rosette-negative fraction after 36 h of culture, using adherence to human Ig-coated plates for depletion of contaminant monocytes and 13.75 g/100 ml metrizamide (Metrizamide AG; GIBCO BRL) for final enrichment in a low-density interface.

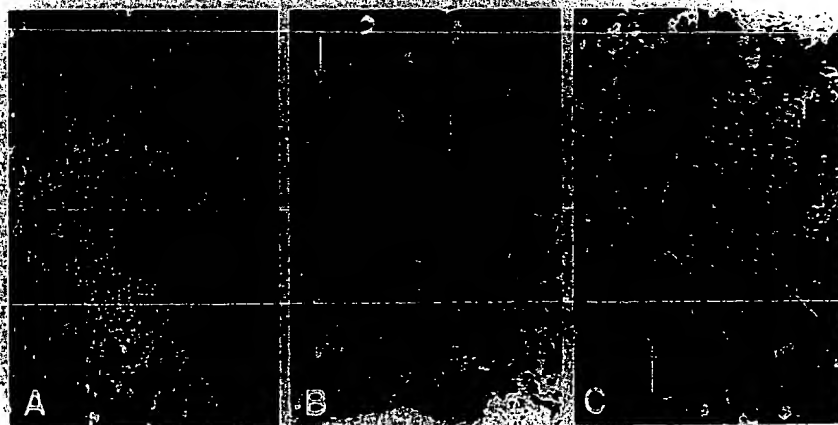
**Allogeneic MLR.** The allogeneic MLR was used as a test for accessory function of marrow-derived APCs, compared with the stimulatory activity of a representative population of mature blood dendritic cells in the same assay. It was not logistically possible to obtain blood dendritic cells and CD34<sup>+</sup> bone marrow progenitors from the same donors, but the T cell responders were always from the same donor in a given experiment. Candidate APCs were washed free of cytokines and irradiated with 1,500 rad <sup>137</sup>Cs (sufficient to prevent continued background proliferation of CD34<sup>+</sup> progeny) before addition in graded doses to responder T cells in 96-microwell tissue culture plates ( $1.5 \times 10^5$  T cells/flat-bottomed microwell or  $1 \times 10^5$  T cells/round-bottomed microwell). Accessory activity of a particular APC population was based on [<sup>3</sup>H]thymidine incorporation ( $1 \mu\text{Ci}$  [<sup>3</sup>H]TdR/microwell; New England Nuclear, Boston, MA) by the responder T cells during an 8–12-h pulse on d 4–5 of the allogeneic MLR. Responses have been expressed as the mean cpm  $\pm$  SD of triplicates. T cells or APCs only ( $1,500 \text{ rad } ^{137}\text{Cs}$ ) always incorporated  $<200$ – $300 \text{ cpm}$ .

## Results

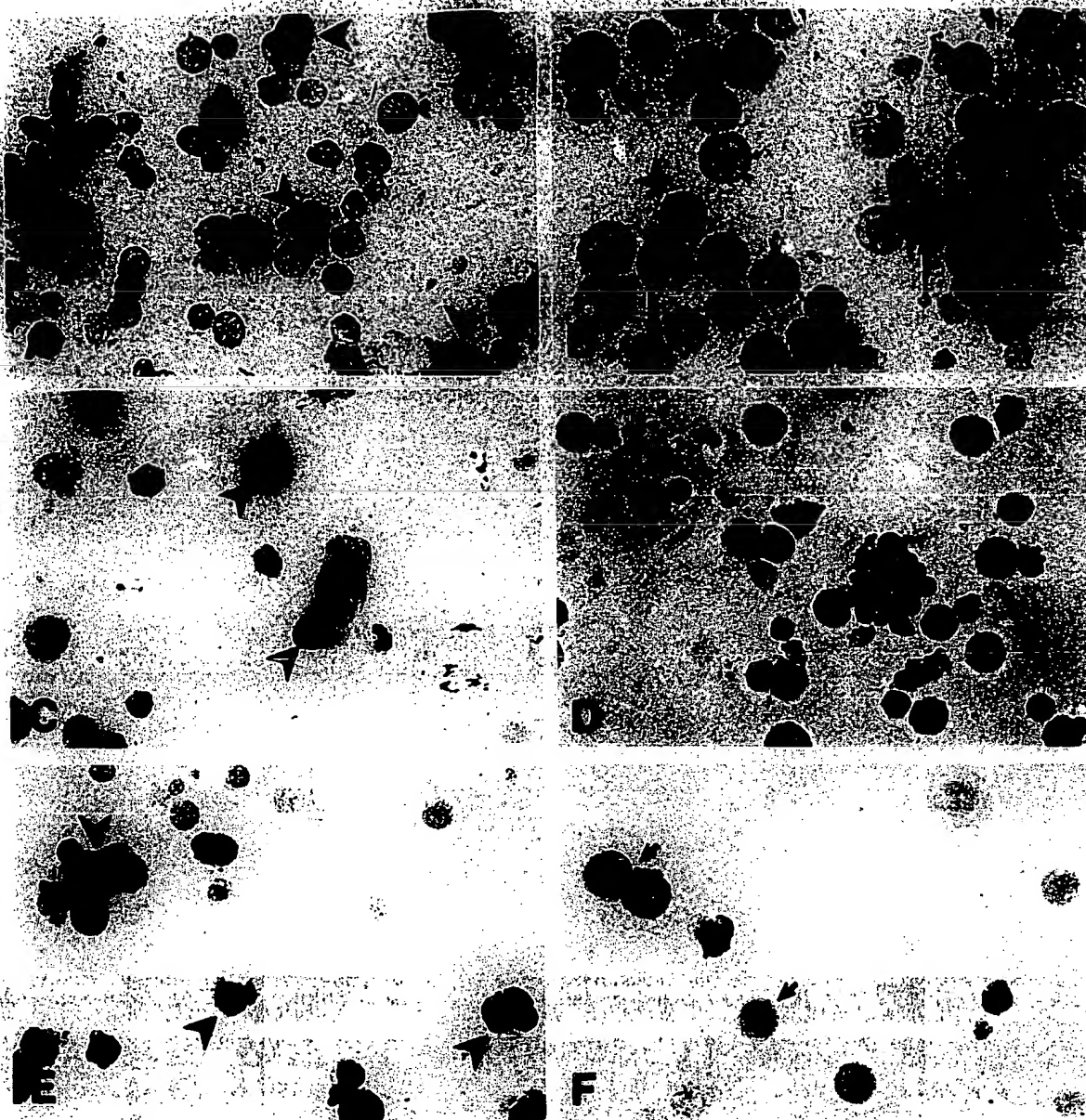
**The Addition of TNF- $\alpha$  to GM-CSF Generates Pure Dendritic Cell Colonies, in Addition to Typical CFU-GM-derived Colonies.** Human CD34<sup>+</sup> bone marrow progenitors were cultured in methylcellulose supplemented with KL and GM-CSF, with or without exogenous TNF- $\alpha$  (1, 2, 8). By direct inspection under phase-contrast microscopy, typical myeloid GM colonies developed irrespective of the addition of TNF- $\alpha$  (Fig. 1 A). When TNF- $\alpha$  was added to the combination of KL and GM-CSF, a morphologically distinct type of colony developed in addition to GM colonies (Fig. 1, B and C). These novel colonies were more loosely aggregated when compared with the compact GM colonies. An individual candidate dendritic cell colony was comprised of cells with very elongated, spiny cytoplasmic processes that were plastic adherent at the undersurface (*arrowheads*), with overlying clusters of cells, many of which extended veiled cytoplasmic processes into different planes of the medium (*arrows*). KL increased the number and size of all colonies but did not alter their gross morphology.

To characterize the cell progeny, individual colonies were harvested, pooled, and resuspended as described after 12–14 d of primary culture in methylcellulose. Figs. 2 and 3 illustrate cytocentrifuged preparations from the two types of colonies. Nonspecific esterase ( $\alpha$ -naphthyl butyrate)-positive cells were identified in both types of colonies grown in KL, GM-CSF, and TNF- $\alpha$ . Cells with a strongly positive, pancellular stain for nonspecific esterase (Fig. 2, A and B, *solid arrowheads*) constituted 10.8% of the cells from the GM colonies and 8.6% of the cells from the putative dendritic cell colonies (500 cell differentials). The candidate dendritic cell colonies also included  $\sim 10\%$  (49 out of 500 cells counted) cells with a dense perinuclear positivity for nonspecific esterase (Fig. 2 B, *short arrows*) and many cells with a faint perinuclear blush (Fig. 2 B, *long thin arrows*). In contrast, myeloperoxidase was limited exclusively to cells in the GM colonies (Fig. 2 C, compare with Fig. 2 D). Acid phosphatase was densely positive in some cells in the GM colonies (Fig. 2 E, *arrowheads*), whereas a minor population of cells stained with a blushlike pattern in the candidate dendritic cell colonies (Fig. 2 F, *short arrows*). Wright-Giemsa staining (not shown) revealed predominantly eosinophils among the granulocytes in the GM colonies, admixed with monocyte/macrophages, the former reflective of the effect of KL. As expected from prior reports investigating the effects of TNF- $\alpha$  on granulopoiesis (17, 18), neutrophilic granulocyte differentiation in GM colonies was also suppressed by TNF- $\alpha$ . The candidate dendritic cell colonies were devoid of granulocytes and included exclusively large cells with cytoplasmic projections, perinuclear clear zones, and folded/lobulated nuclei, all of which are characteristic of blood dendritic cells.

Immunophenotyping is illustrated in Fig. 3. All of the cells from the candidate dendritic cell colonies were intensely positive for HLA-DR/DQ and exhibited the prominent cytoplasmic veils (Fig. 3 A, *arrows*) that typify mature dendritic cells. The CFU-GM-derived progeny included some what less strongly positive cells for class II MHC that lacked cytoplasmic projections (Fig. 3 B), although even a rare positive cell with the veiled dendritic morphology illustrated



**Figure 1.** CD34<sup>+</sup> bone marrow progenitors cultured in methylcellulose/IMDM-30% FCS, KL, GM-CSF, and TNF- $\alpha$  yield novel dendritic cell colonies, in addition to typical GM myeloid colonies. CD34<sup>+</sup> bone marrow progenitors cultured for 12–14 d with 20 ng/ml KL, 100 ng/ml GM-CSF, and 10 ng/ml TNF- $\alpha$ , in 1.2% methylcellulose/IMDM-30% FCS, developed into two morphologically distinct types of colonies: (A) typical GM colonies ( $\times 65$ ); and (B and C) separate but more loosely aggregated dendritic cell colonies comprised of plastic adherent cells (*arrowheads*) with elongated, spiny cytoplasmic processes at the undersurface, and overlying cells (*arrows* highlight a few of these) extending veils into different planes of the medium (B,  $\times 130$ , and C,  $\times 65$ , photographed at different planes in the methylcellulose culture).



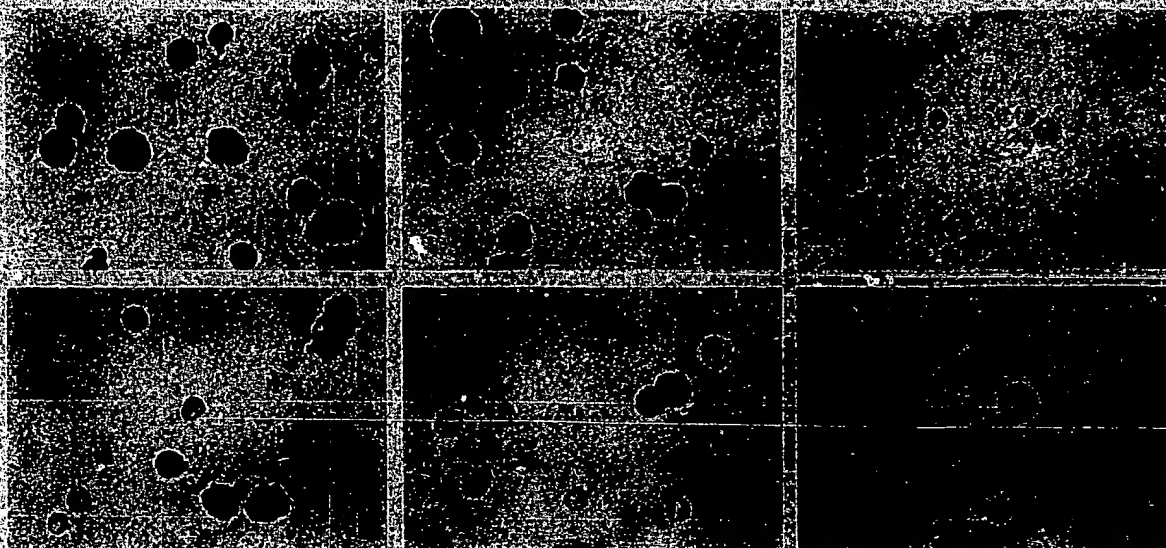
**Figure 2.** Cytochemical staining of the clonogenic progeny of CFU-GM and CFU-DIC. CD34<sup>+</sup> bone marrow progenitors cultured for 12–14 d with 20 ng/ml KL, 100 ng/ml GM-CSF, and 10 ng/ml TNF- $\alpha$  in 1.2% methylcellulose/IMDM–30% FCS, developed into the two distinct types of colonies depicted in Fig. 1. These were plucked under direct microscopic inspection, pooled, and resuspended into single-cell suspensions. CFU-GM progeny are depicted in A, C, and E; CFU-DIC progeny are shown in B, D, and F. (A and B)  $\alpha$ -naphthyl butyrate esterase (X280); (C and D) myeloperoxidase (X250); (E and F) acid phosphatase (X250). See text for arrow descriptors. In situ staining of colonies in agarose plates confirmed these cytochemical profiles (not shown).

in Fig. 3 A could be identified ( $\leq 1\%$  of the total cells; not shown in Fig. 3 B). CD1a<sup>+</sup> cells were also identified in both types of colonies (Fig. 3, C and D), although they constituted a very small fraction of the cells in the GM colonies (Fig. 3 D; the field shown is not typical but was selected to illustrate the presence of positive cells). CD14 completely segregated the two kinds of colonies. True CD14<sup>+</sup> cells were limited exclusively to the CFU-GM-derived progeny (Fig. 3 F) and were comparable in frequency

to the percentage of nonspecific esterase-positive cells in these GM colonies (Fig. 2 A). More than 98–99% of the cells in the candidate dendritic cell colonies were clearly CD14<sup>−</sup> with a rare cell ( $\leq 1$ –2%) staining very faintly at most (Fig. 3 E). CD14<sup>dim</sup> cells were not found in the GM colonies.

*CFU-GMs and CFU-DICs Represent Distinct Clonogenic Progenitors.* CD34<sup>+</sup> bone marrow cells were cultured in 0.36% agarose/IMDM–20% FCS, supplemented with the exoge-





**Figure 3.** Immunophenotype of the clonogenic progeny of CFU-GM and CFU-DC.  $CD34^+$  bone marrow progenitors cultured for 12–14 d with 20 ng/ml KL, 100 ng/ml GM-CSF, and 10 ng/ml TNF- $\alpha$  in 1.2% methylcellulose/IMDM–30% FCS, developed into the two distinct types of colonies depicted in Fig. 1. These were plucked under direct microscopic inspection, pooled, and resuspended into single-cell suspensions. A, C, and E are CFU-DC-derived progeny, and B, D, and F are CFU-GM-derived progeny. (A and B) HLA-DR/DQ staining by 9:3C9, with arrows highlighting the unique, very fine cytoplasmic processes of dendritic cells (these may be more easily visualized on the printed page using hand-held magnification); (C and D) CD1a staining by OKT6; (E and F) CD14 staining by 3C10, with a typical monocyte in the GM colonies indicated by an arrow in F (the punctate staining in the other small cells is background staining of eosinophil granules).  $\times 165$ .

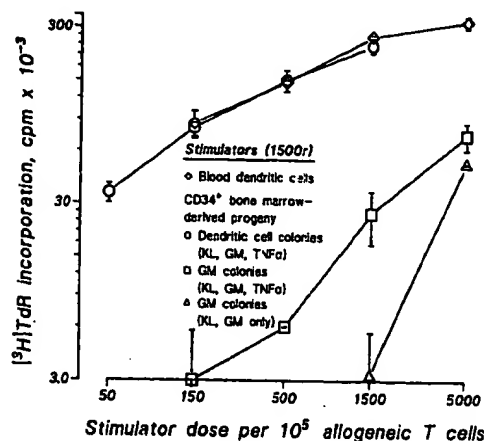
**Table 1.** Primary Cloning Efficiency of  $CD34^+$  Human Bone Marrow Cells

Condition number	Cytokine stimuli	Number of experiments	GM colonies <sup>‡</sup>		Dendritic cell colonies <sup>‡</sup>		
			Absolute yield	Percent cloning efficiency	Absolute yield	Percent cloning efficiency	Percentage of total colonies
1	None	4	29 $\pm$ 29	0.03	1 $\pm$ 1	0.001	3.3
2	20 ng KL + 50 ng IL-3 + 20 ng IL-6 $\pm$ 5 U epo.	6	7,880 $\pm$ 1,754	7.9	3 $\pm$ 3	0.003	0.03
3	10 ng TNF- $\alpha$	3	5 $\pm$ 5	0.005	0 $\pm$ 0	0	0
4	100 ng GM-CSF	6	1,667 $\pm$ 239	1.7	16 $\pm$ 7	0.016	0.96
5	100 ng GM-CSF	6	1,576 $\pm$ 500	1.6	1,176 $\pm$ 165	1.2	42.7
6	20 ng KL $\pm$ 100 ng GM-CSF + 10 ng TNF- $\alpha$ <sup>§</sup>	7	2,852 $\pm$ 970	2.9	2,031 $\pm$ 224	2	41.6

\*Cytokine doses are per milliliter.

<sup>‡</sup>Colony counts are adjusted to  $10^5$   $CD34^+$  bone marrow progenitors cultured at  $1-2 \times 10^3$ /ml in 0.36% agarose/IMDM–20% FCS for 12–14 d; the yields represent means  $\pm$  SEM of triplicate plates per condition per experiment.

<sup>§</sup>A dose response evaluation of TNF- $\alpha$  added to 20 ng/ml KL and 100 ng/ml GM-CSF demonstrated an increasing yield of dendritic cell colonies with increasing amounts of TNF- $\alpha$ . KL, GM-CSF + 1 ng/ml TNF- $\alpha$ : 150  $\pm$  30 DC colonies; + 2.5 ng/ml TNF- $\alpha$ : 450  $\pm$  100 DC colonies; + 5 ng/ml TNF- $\alpha$ : 850  $\pm$  50 DC colonies; + 10.0 ng/ml TNF- $\alpha$ : 4,300  $\pm$  400 DC colonies (triplicate means  $\pm$  SEM,  $n = 1$  experiment).



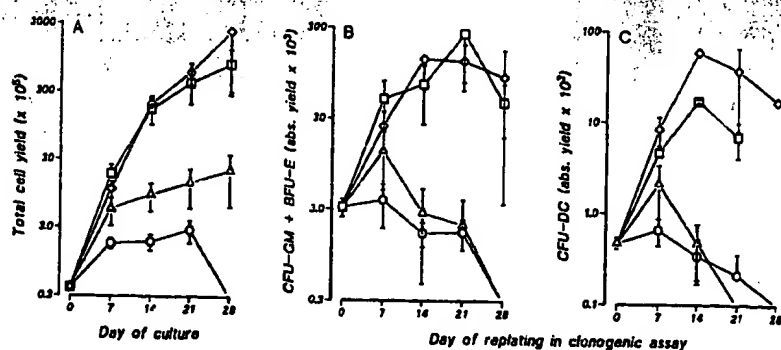
**Figure 4.** CD34<sup>+</sup> bone marrow progenitors, grown in colony assays under serum-replete conditions with KL, GM-CSF, and TNF- $\alpha$ , generate potent immunostimulatory progeny in dendritic cell colonies but not in colonies derived from typical myeloid CFU-GM. CD34<sup>+</sup> bone marrow progenitors were cultured for 12–14 d with 20 ng/ml KL, 100 ng/ml GM-CSF, with or without 10 ng/ml TNF- $\alpha$ , in 1.2% methylcellulose/IMDM–30% FCS. Colonies were plucked under direct microscopic inspection, pooled, and resuspended into single-cell suspensions. Stimulatory capacity was assessed in primary allogeneic MLRs and compared with that effected by blood dendritic cells as a positive control. All stimulator populations were exposed to 1,500 rad <sup>137</sup>Cs and then added in graded doses to 10<sup>5</sup> allogeneic T cells in U-bottomed microwells. Cells were pulsed with 1  $\mu$ Ci/well [<sup>3</sup>H]TdR from 92 to 104 h. Circles denote dendritic cell colonies; squares denote GM colonies grown in the presence of KL, GM-CSF, and TNF- $\alpha$ ; triangles denote GM colonies grown in KL and GM-CSF only; and diamonds denote blood dendritic cell comparison. Results are representative of three experiments. Please note log scale.

nous cytokines shown in Table 1, and scored after 12–14 d. Based on six experiments performed in triplicate, CD34<sup>+</sup> bone marrow progenitors yielded similar numbers of GM colonies in the presence of GM-CSF, with or without TNF- $\alpha$ . With the addition of TNF- $\alpha$ , dendritic cell colonies developed in addition to GM colonies. This effect of

TNF- $\alpha$  was dose related but plateaued between 10 and 100 ng/ml (not shown). KL affected the primary cloning efficiency of all CD34<sup>+</sup> progenitors by doubling the number and increasing the size of both types of colonies.

**Dendritic Cell Colonies Are Comprised of Potent Immunostimulatory Cells for the Initiation of Primary Allogeneic Responses by Resting T Cells.** After the two colony types were harvested from methylcellulose, single-cell suspensions were tested as stimulators of resting allogeneic T cells. As shown in Fig. 4, the capacity of cells derived from the candidate dendritic cell colonies to stimulate an allogeneic MLR was identical to that of mature blood dendritic cells. This confirmed the substantial expansion and enrichment of dendritic cells in these unique colonies. Cells from GM colonies generated in the presence of KL, GM-CSF, and TNF- $\alpha$  exerted weak but measurable stimulatory activity, in accordance with the  $\leq 1\%$  candidate dendritic cells seen by class II MHC staining of these colonies. Their stimulatory capacity was nevertheless  $\sim 1.5$  logs less potent than the cells from dendritic cell colonies, and cells from GM colonies grown in the absence of TNF- $\alpha$  were  $>2$  logs less potent.

**KL Synergizes with GM-CSF and TNF- $\alpha$  to Recruit and Expand the Pool of Clonogenic Progenitors (CFUs) Responsive to GM-CSF and TNF- $\alpha$ -induced Dendritic Cell Expansion and Differentiation.** To quantify expansion of committed progenitors, CD34<sup>+</sup> bone marrow cells were cultured in suspension in IMDM–20% FCS with the cytokine combinations shown in Fig. 5. At 7-d intervals, these suspension cultures were harvested, washed, and either (A) replated in liquid suspension at the initial starting concentration ( $4 \times 10^4$  cells/ml) with the same cytokines for an additional 7 d of cell expansion, or plated in 0.36% agarose/IMDM–20% FCS for 12–14 d with (B) a positive control combination of KL, IL-3, IL-6, and epo to quantify expansion of CFU-GM and erythroid burst-forming unit, or with (C) GM-CSF and TNF- $\alpha$  to quantify expansion of dendritic cell colonies.



**Figure 5.** Progenitor expansion and replating efficiency of human CD34<sup>+</sup> bone marrow cells; effect of KL. CD34<sup>+</sup> bone marrow cells were cultured in IMDM–20% FCS suspension cultures supplemented with the following cytokines: no exogenous cytokine (circles); KL, IL-3, IL-6, and epo (squares); GM-CSF and TNF- $\alpha$  (triangles); and KL, GM-CSF, and TNF- $\alpha$  (diamonds). Cytokine doses were as follows: 20 ng/ml KL, 100 ng/ml GM-CSF, 10 ng/ml TNF- $\alpha$ , 50 ng/ml IL-3, 20 ng/ml IL-6, and 5 U/ml epo. (A) Weekly expansion of total cell numbers in suspension culture with the respective cytokine conditions, adjusted to a starting cell dose of  $4 \times 10^4$  per condition. (B and C) Cells expanded in suspension culture with exogenous cytokines (shown in A) were harvested at weekly intervals. One cohort was returned to suspension culture for continued expansion. Another cohort of cells was cultured in IMDM–20% FCS–0.36% agarose, (B) supplemented with KL, IL-3, IL-6, and epo to determine the numbers of CFU-GM + erythroid burst-forming units that had been expanded in suspension based on colony growth in semisolid medium; or (C) supplemented with GM-CSF and TNF- $\alpha$  to quantify expansion of CFU-DC based on subsequent dendritic cell colony growth in semisolid medium. Colonies were enumerated 12–14 d after secondary replating. Baseline or “input” CFUs were determined by colony growth from day 0 in the respective cytokines. CFU yields have been adjusted to a starting cell dose of  $4 \times 10^4$  CD34<sup>+</sup> bone marrow progenitors per condition. Means are derived from pooled data from three experiments, with each condition performed in triplicate. Error bars represent the SEM. Similar results were obtained in three additional experiments. Please note log scale. Also note that all symbols refer to primary suspension culture conditions in A.

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KL expanded the pool of committed clonogenic progenitors responsive to GM-CSF- and TNF- $\alpha$ -induced dendritic cell differentiation by almost 100-fold during the first and second weeks of suspension culture. This was determined by dividing the number of dendritic cell colonies generated in agarose after weekly expansion in suspension culture with KL, GM-CSF, and TNF- $\alpha$  by the baseline number of dendritic cell colonies generated by GM-CSF and TNF- $\alpha$  from day 0 of CD34<sup>+</sup> isolation. Even with primary exposure to a non-dendritic cell-selective cytokine combination, like KL, IL-3, IL-6, and epo, progenitors with the capacity to generate dendritic cells when secondarily exposed to GM-CSF and TNF- $\alpha$  expanded almost 30-fold over baseline. For purposes of comparison, KL, IL-3, IL-6, and epo expanded CFU-GM  $\sim$ 80-fold by day 21; and KL, GM-CSF, and TNF- $\alpha$  expanded CFU-GM  $\sim$ 40-fold by days 14 and 21. Progenitors responsive to GM or dendritic cell colony-differentiating cytokines were maintained for several weeks in suspension culture by KL, although absolute CFU numbers declined after 14–21 d. In the absence of KL, the number of progenitors increased minimally during the first week of suspension culture but declined to almost undetectable levels thereafter.

## Discussion

Using a combination of morphologic, phenotypic, and functional criteria, we have identified a novel hematopoietic pathway for the generation of pure dendritic cell colonies. These dendritic cell colonies develop from normal human CD34<sup>+</sup> bone marrow progenitors under serum-replete conditions supplemented by GM-CSF and TNF- $\alpha$ . Dendritic cell colonies are distinct from and develop in addition to typical myeloid GM colonies under these same cytokine conditions. GM-CSF, in the absence of TNF- $\alpha$ , does not support growth of dendritic cell colonies. As is true of other hematopoietic progenitor populations, KL enhances by approximately twofold the primary cloning efficiency of dendritic cell progenitors. KL also expands the pool of committed clonogenic precursors for dendritic cell colonies by almost 100-fold during the first and second weeks of culture.

These pure human dendritic cell colonies differ from previously described myeloid colonies containing small numbers of dendritic cells (2, 5, 19). Inaba et al. generated mixed myeloid colonies from class II MHC negative murine bone marrow precursors in the presence of GM-CSF and 10% FCS (5). These colonies included 0.5–1.5% dendritic cells, indicating that murine phagocytes (granulocytes and macrophages) share a common MHC class II negative precursor with dendritic cells at some point in their development. Reid et al. reported a clonable dendritic cell progenitor in both human marrow and blood (19). They further demonstrated that this precursor was enriched among the CD34<sup>+</sup> population and dependent upon GM-CSF and TNF- $\alpha$  (2). However, the resultant colonies had relatively few dendritic cells (4–50 dendritic cells per colony) and included additional cells with macrophage morphology. When

they cultured a heterogeneous population of nonadherent BMMNC in methylcellulose with GM-CSF, IL-3, with or without G-CSF, and TNF- $\alpha$  or TNF- $\beta$ , rare candidate colonies of pure dendritic cells were described (2). Dendritic cell colonies were identified solely by morphology and CD1a expression, however, and their cloning efficiency was  $<0.05\%$  from this undefined progenitor population (2). Limited cell yields did not permit additional phenotypic characterization or functional comparison with known dendritic cells to assess stimulatory activity.

It should be noted that CD1a is not unique to thymocytes and Langerhans/dendritic cells, however, as cytokines like GM-CSF and IL-4 can induce expression of CD1 epitopes on monocytes (20, 21). Our own data corroborate this finding, based on the occasional CD1a<sup>+</sup> cells noted in the GM colonies. Other single criteria could be equally misleading, e.g., nonspecific esterase, which is not only present in mature monocytes but also in resident Langerhans cells, in which it diminishes with maturation in culture (22). CD14 and myeloperoxidase definitively discriminated the two types of colonies, with positives found only in the GM colonies. Cells in 12–14-d dendritic cell colonies were clearly CD14 and myeloperoxidase negative. A rare cell that stained very faintly with anti-CD14 could be identified in dendritic cell, but not GM, colonies; this is consistent with prior reports of freshly isolated blood, resident epidermal, or cytokine-generated marrow-derived dendritic cells (8, 23–25). The combination of these morphologic and phenotypic criteria therefore distinguished two types of colonies, each of which developed alongside the other from normal CD34<sup>+</sup> progenitors when TNF- $\alpha$  was added to GM-CSF with or without KL under serum-replete conditions.

These clonogenic progeny also exhibited divergent properties for the stimulation of resting primary T cells. We used the allogeneic mixed leukocyte reaction for this purpose, because it highlights the specialized accessory function of mature dendritic cells vs other APCs for the initiation of T cell-mediated immune responses (for review see reference 26). The stimulatory capacity of dendritic cell progeny generated in colonies with KL, GM-CSF, and TNF- $\alpha$  was equivalent to that of mature blood dendritic cells and exceeded that of GM colony-derived cells by at least 1.5–2 logs. Interestingly, the GM colonies that arose in the presence of TNF- $\alpha$  exerted low but measurable stimulatory activity that was greater than that effected by GM colonies grown in the absence of TNF- $\alpha$ . Even a trace growth of dendritic cells among the human GM progeny generated in the presence of GM-CSF and TNF- $\alpha$ , vs GM-CSF alone, could account for this level of stimulation. This would be in accordance with the murine system (5) and is supported by our observation of  $\leq 1\%$  candidate dendritic cells by Wright-Giemsa and class II MHC staining of these particular GM colonies. The alternative possibility of contamination with cells from adjacent dendritic cell colonies during manual harvesting cannot be excluded.

Substantial expansion by KL was demonstrated for both GM and dendritic cell progenitors in secondary colony as-



says performed weekly after expansion in IMDM-20% FCS and cytokines. Bone marrow CD34<sup>+</sup> dendritic cell progenitors expanded almost 100-fold during the first and second weeks of primary suspension culture in KL, GM-CSF, and TNF- $\alpha$ . Even KL with IL-3, IL-6, and epo in primary suspension cultures effected a 25-fold expansion of GM-CSF/TNF- $\alpha$ -responsive dendritic cell progenitors in secondary clonogenic assays. These degrees of progenitor expansion are in marked contrast to that supported by GM-CSF and TNF- $\alpha$  in the absence of KL. Furthermore, although absolute colony numbers declined after 2 wk, progenitors responsive to differentiating cytokines were maintained by KL for several weeks of in vitro culture.

The development of dendritic cell colonies, without alteration in the growth of CFU-GM, indicates that CD34<sup>+</sup> bone marrow cells include a dendritic cell progenitor that is distinct from CFU-GM. DC colonies do not result from TNF modification of committed CFU-GM, as the latter

remain constant in number and appearance with or without TNF- $\alpha$ . This CFU-DC is KL responsive, increasing its cloning efficiency and expanding almost 100-fold during the first 2 wk of serial cultures. Dendritic cell colonies of this frequency and purity have not been previously reported. Mixed myeloid colonies with trace numbers of dendritic cells reflect the small proportion of dendritic cells in most tissues where they are identified in vivo (for review see reference 26). Epidermis and afferent lymph, however, contain many dendritic cells but few if any monocytes/macrophages. The CFU-DCs that we have described may constitute an active hematopoietic pathway for generating dendritic cell populations in these sites in vivo under steady-state conditions. This pathway may have the additional role of increasing the numbers of these specialized APCs in response to inflammatory cytokines during T cell-mediated immune responses.

Maureen Sullivan merits special thanks for her expert assistance with the colony assays, as do Kayo Inaba and Ralph Steinman for their constructive advice and criticism. We also appreciate the technical assistance of Koji Shido, Liang Jin, and Judy Adams. We thank Susan McKenzie and her laboratory staff for their assistance with Wright-Giemsa and cytochemical staining. We also acknowledge the physicians, Sharon Bleau, and Dr. Nancy Collins of the Allogeneic Bone Marrow Transplantation Service, Memorial Sloan-Kettering Cancer Center, for their help in obtaining the bone marrow samples for these studies.

This work was supported by grants AI-26875 (J. W. Young), CA-23766 (J. W. Young), AI-01254 (P. Szabolcs), HL-46546 (M. A. S. Moore), and CA-08748 (M. A. S. Moore) from the National Institutes of Health; and IM-757 (J. W. Young) from the American Cancer Society. P. Szabolcs was also the Robert Schwartz Fellow for Pediatric Cancer Research, Memorial Sloan-Kettering Cancer Center, and was additionally supported in part by the Toys 'R' Us/Friends of the Children Foundation.

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Received for publication 19 October 1994 and in revised form 13 April 1995.

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